

EXHIBIT 5

Fetal DNA in maternal plasma is elevated in pregnancies with aneuploid fetuses

Xiao Yan Zhong¹, Martin R. Bürk¹, Carolyn Troeger¹, Laird R. Jackson², Wolfgang Holzgreve¹ and Sinuhe Hahn^{1*}

¹Laboratory for Prenatal Medicine, Department of Obstetrics and Gynaecology, University of Basel, Switzerland

²Division of Medical Genetics, Jefferson Medical College, Philadelphia, USA

Current non-invasive screening methods for the prenatal diagnosis of fetal aneuploidies are hampered by low sensitivities and high false positive rates. Attempts to redress this situation include the enrichment of fetal cells from maternal blood, or the use of fetal DNA in the plasma of pregnant women. By the use of real-time quantitative polymerase chain reaction (PCR) it has recently been shown that circulatory male fetal DNA in maternal plasma is elevated in pregnancies with trisomy 21 fetuses. In this independent study we confirm and extend upon these results by showing that the levels of fetal DNA are also elevated in pregnancies with other chromosomal aneuploidies (mean=185.8 genome equivalents/ml; range=62.2–471.7) when compared to pregnancies with normal male fetuses (mean=81.9 genome equivalents/ml; range=28.8–328.9), $p=0.005$. This elevation was greatest for fetuses with trisomy 21, whereas it was not significant for fetuses with trisomy 18, $p=0.356$. These data suggest that a quantitative analysis of such fetal DNA levels may serve as an additional marker for certain fetal chromosomal abnormalities, in particular for trisomy 21. Copyright © 2000 John Wiley & Sons, Ltd.

KEY WORDS: non-invasive; prenatal diagnosis; aneuploidy screening

INTRODUCTION

Modern genetic counsellors are faced by the dilemma that there is an increasing demand for prenatal diagnosis, but that couples are reluctant to face the risk imposed on mother and child by invasive procedures such as amniocentesis or chorion villus sampling (Holzgreve, 1997). Due to the low sensitivity of current non-invasive screening methods, such as serum analytes or ultrasound, a high proportion of aneuploid fetuses are not successfully detected early in pregnancy. Furthermore, since these methods have high false positive rates (over 5%), a large number of unnecessary invasive practices are performed, which apart from the associated risk, place considerable psychological distress on the couples involved (Wald *et al.*, 1999). It is for this reason that several concerted efforts are underway to develop an efficient non-invasive method for prenatal diagnosis, one of the most promising of which is the enrichment of fetal cells from maternal blood (Hahn *et al.*, 1998; Bianchi, 1999). The efficacy of the latter for the detection of fetal aneuploidies is currently the subject of a large scale clinical study conducted under the auspices of the National Institute of Child Health and Development (NICHD) (de la Cruz *et al.*, 1995), in which our laboratories are participating. Although, preliminary data from this study have indicated that considerably lower false rates for the specific detection of fetal

aneuploidies can be attained than with other non-invasive methods (de la Cruz *et al.*, 1998; Bianchi *et al.*, 1999), this methodology is currently too cumbersome and labour intensive to be used for wide-scale screening (Bischoff *et al.*, 1998; Oosterwijk *et al.*, 1998; Hahn *et al.*, 1999).

A novel approach for the non-invasive prenatal diagnosis of fetal genetic characteristics is by the use of free extracellular fetal DNA which can be detected by polymerase chain reaction (PCR) in the serum or plasma of pregnant women (Lo *et al.*, 1997). This approach has successfully been used for the prenatal diagnosis of fetal sex and rhesus D status (Faas *et al.*, 1998; Lo *et al.*, 1998b; Hahn *et al.*, 2000; Zhong *et al.*, 2000). By the use of real-time quantitative PCR technology (Lo *et al.*, 1998a), elevations in the amount of this fetal DNA have very recently been observed in pregnancies with trisomy 21 fetuses (Lo *et al.*, 1999a). We have attempted to confirm this observation and also whether it can be extended to other fetal aneuploidies.

MATERIAL AND METHODS

Patients

Pregnant women with an increased risk for a aneuploid fetus and who were about to undergo an invasive prenatal diagnostic procedure were recruited for this study. Informed consent was obtained in each instance, as was ethical approval by our institutional review committees.

*Correspondence to: S. Hahn, Laboratory for Prenatal Medicine, Department of Obstetrics and Gynecology, University of Basel, Schanzenstrasse 46, CH 4031 Basel, Switzerland.
E-mail: shahn@uhbs.ch

Samples

This study was performed in a retrospective manner. Twenty-eight plasma samples were selected from pregnancies with a singleton male aneuploid fetus. In parallel, 29 samples were taken from normal pregnancies with a male fetus. The average gestational age at which the samples were taken was 14+4 weeks. The samples were blinded to all personnel involved with preparation and analysis.

DNA extraction

The blood samples, 15 ml on average, were processed within 24 h of sampling. Following separation of the plasma by centrifugation at 1200 g for 10 min, 1.5 ml plasma aliquots were removed and stored at -80°C . For analysis, DNA was extracted from 400 μl plasma using the QIAamp Blood Kit (Qiagen, Basel, Switzerland) according to the manufacturer's protocol. The DNA extraction was carried out by female staff, to minimize the possibility of contamination. In addition to multiple negative controls being included in each analysis, aerosol resistant tips (ART, Molecular Bio-Products, San Diego, California, USA) were used throughout, as was UV-cross-linking of the relevant pipetting instruments (Stratagene Stratalinker, San Diego, California, USA). The DNA preparations were eluted in 50 μl elution buffer (10 mM Tris HCL pH 7.4: 1 mM EDTA), of which 2 μl was used as a template for the PCR reaction.

TaqMan PCR analysis

The TaqMan real time PCR analysis was performed using a PE Applied Biosystems 7700 Sequence Detector for which primers and dual labelled probes which had been designed with the aid of Primer Express software (Perkin Elmer, Branchburg, New Jersey, USA) were used. To determine the amount of male fetal DNA the Y chromosome SRY locus (GenBank Accession Nr. L08063) was used. The size of the fragment analysed was 78 bp. The primer and probe combination used were:

SRY:

(forward): 5' TCC TCA AAA GAA ACC GTG CAT 3'
(reverse): 5' AGA TTA ATG GTT GCT AAG GAC TGG AT 3'

SRY probe: 5' (FAM) CAC CAG CAG TAA CTC CCC ACA ACC TCT TT (TAMRA) 3'

Since the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GenBank Accession Nr. J04038) gene is common to all genomes, this primer/probe combination was used to determine the total amount of DNA present in the plasma, and served as a control that DNA was indeed present in the sample analysed. The length of the fragment analysed was 97 bp.

GAPDH:

(forward): 5' CCC CAC ACA CAT GCA CTT ACC 3'
(reverse): 5' CCT AGT CCC AGG GCT TTG ATT 3'
GAPDH probe 5' (FAM) AAA GAG CTA GGA AGG ACA GGC AAC TTG GC (TAMRA) 3'

For the TaqMan PCR analysis we used 25 μl reaction volumes containing 2 μl of the extracted DNA, 300 nM of each amplification primer and 100 nM of the dual-labelled TaqMan probe and the necessary components provided in the TaqMan PCR Core reaction Kit (Perkin Elmer, Branchburg, New Jersey, USA). This corresponded to 2.5 μl of $10\times$ Buffer A, 3.5 mM MgCl_2 , 100 mM dNTPS, 0.025 U AmpliTaq Gold and 0.01 U Amp Erase. The uracil *N*-glycosylase activity of latter, in combination with dUTP, was used to prevent contamination by the carry-over of PCR products.

Since we had designed the analysis in such a manner that identical thermal profiles were used for both the SRY and GAPDH TaqMan assay, the DNA were analysed for these two markers on the same plate in the same analytic run. These were carried using a 2 min incubation at 50°C (to permit Amp Erase activity), followed by an initial denaturation step at 95°C for 10 min, which facilitates activation of the AmpliTaq Gold polymerase activity, followed by 40 cycles of 1 min at 60°C and 15 s at 95°C .

To determine the number of copies of male DNA present in the plasma sample a standard dilution curve using a known concentration of male genomic DNA was used. For the conversion to the number of copies or genomes equivalents, 6.6 pg was used as described previously (Lo *et al.*, 1998a). All samples were analysed in duplicate and scored in a blinded manner.

Statistics

Since the data were parametric the two tailed *t*-test was performed, using the SPSS software package for Windows. Data are represented as scatter diagrams and include mean values.

RESULTS

The TaqMan real-time quantitative PCR system has recently been used to quantify the amount of fetal DNA in normal pregnancies (Lo *et al.*, 1998a) and in those affected by preeclampsia (Lo *et al.*, 1999b; Holzgreve and Hahn, 1999), where it has been shown to be both accurate and reliable. We have now made a similar analysis regarding the amount of fetal DNA in pregnancies bearing an aneuploid fetus.

In order to verify the specificity of this assay in our hands, we first examined 30 samples taken from women currently pregnant with a female fetus, 12 of which had previously borne a male fetus. No Y chromosome positive signals were detected in any of these samples, indicating a specificity of 100% for the Y chromosome specific PCR assay. In a comparable analysis of samples obtained from women pregnant with male fetuses, we were able to detect SRY specific

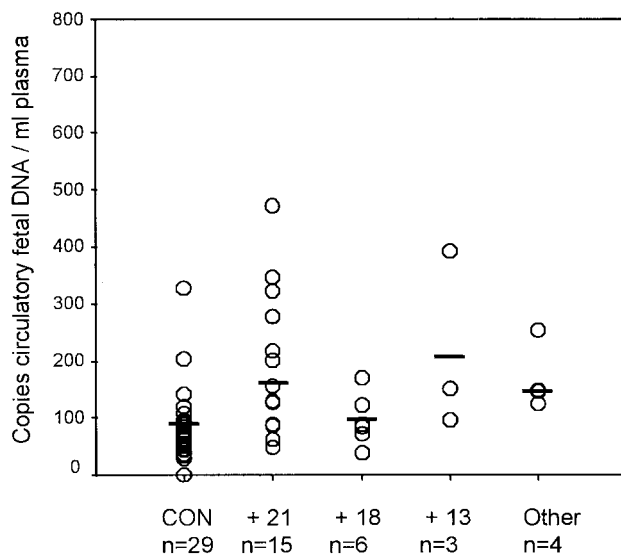


Figure 1—Levels of fetal DNA in maternal plasma in normal and aneuploid pregnancies

PCR amplicons in 34 out of the 36 samples examined. This indicated a sensitivity of 94.4% for the SRY specific PCR assay. Amplicons for the control GAPDH gene, indicating the presence of DNA, were detected in all the samples examined (Zhong *et al.*, in press). This implies that fetal DNA may not be detectable in all the samples analysed, confirming previous reports (Lo *et al.*, 1998b).

In the analysis for this report, which was performed in a blinded manner, our data demonstrate that the mean fetal DNA levels are significantly elevated in the 15 plasma samples analysed from fetuses with trisomy 21 (mean = 185.7 copies/ml of maternal plasma) when compared to pregnancies with normal male fetuses (mean = 83.1 copies/ml of maternal plasma) (Figure 1 and Table 1).

Our analysis of pregnancies with other fetal chromosomal aneuploidies showed that fetal plasma DNA levels were not significantly elevated from the norm in fetuses with trisomy 18 (95.5 copies/ml compared with 83.1 copies/ml) (Figure 1 and Table 1).

For the three cases with trisomy 13 and the four other chromosomal aberrations examined, it appears that the levels of fetal DNA are also elevated as a mean of 213.2 and 168.7 copies of fetal DNA per ml maternal plasma were detected, respectively. Even

though we were only able to examine two cases with 47+XXY, it is interesting to note that the levels of fetal DNA detected here (125.3 and 146.9 copies/ml of maternal plasma, respectively) were both above the mean detected in the control samples.

DISCUSSION

Our data show that the amount of fetal DNA in maternal plasma is significantly elevated in pregnancies with certain fetal aneuploidies. This effect is particularly striking when comparing normal pregnancies to those with trisomy 21 fetuses, thereby confirming the recent report by Lo and colleagues (Lo *et al.*, 1999a). Our data also suggest that the levels of circulatory fetal DNA may be elevated in pregnancies with other fetal chromosomal aneuploidies, such as the three cases with trisomy 13 or the two with Klinefelter syndrome examined. Due to the small sample size examined we cannot, however, draw any conclusions with regard to these at present. It should also be noted, however, that for several of the samples examined a considerable degree of overlap existed with the corresponding values obtained from the control samples. This result implies that fetal DNA levels are not elevated *per se* in pregnancies with trisomy 21 fetuses or other cases of fetal aneuploidy. No significant elevation in fetal circulatory DNA was detected in six pregnancies with trisomy 18.

Although we have observed slightly higher fetal DNA levels than those reported by Lo and colleagues (Lo *et al.*, 1999a), these could be a reflection of a difference in the DNA extraction protocols, the choice of different PCR primers and probes and the variations which are likely to occur between two separate laboratories. Our study does, however, in an independent and blinded manner confirm the previous observation in that we also observed an almost two fold elevation in the amount of fetal circulatory DNA in pregnancies with 47XY+21 fetuses when compared to pregnancies with normal male fetuses. This result suggests that real time PCR quantitation of circulatory fetal DNA for diagnostic applications could be performed by different laboratories, but that it would probably be most practical to use a multiples of the median (MoM) system akin to that which is currently used for the analysis of serum analytes (Wald *et al.*, 1999).

Table 1—Quantitative analysis of fetal DNA levels in normal pregnancies and those bearing aneuploid fetuses

Fetal genotype	Number of cases	Levels of free fetal DNA per ml maternal plasma	<i>p</i> (relative to control group)
46XY	29	83.1 (0–328.9)	—
47XY+21	15	185.6 (48.7–471.7)	0.005
47XY+18	6	95.5 (37.5–168.9)	0.356
47XY+13	3	213.2 (95.2–392.6)	0.017
47XXY	2	136.2 (125.3–146.9)	—
48XY+18+12	1	71.5	—
69XXY	1	253.1	—

An interesting facet of these data is that they do parallel previous observations regarding fetal cells enriched from maternal blood, where increased numbers of fetal cells were observed in pregnancies with an aneuploid fetus—in particular those with trisomy 21 (Gänshirt-Ahlert *et al.*, 1993; Al-Mufti *et al.*, 1999). Furthermore, our data correlate well with a report by Bianchi and colleagues (Bianchi *et al.*, 1997), who using a different quantitative PCR technique, directly assessed the number of fetal cells in blood of pregnant women. In their study a 4- to 5 fold increase in the number of fetal cells was observed when comparing pregnancies with trisomy 21 fetuses with normal pregnancies. In a similar manner, they also found a 2-fold increase in the number of fetal cells for fetuses with trisomy 13, but none for those with trisomy 18.

The difference in the levels of fetal circulatory DNA for fetuses with trisomy 21 compared to trisomy 18 could in part be explained by the placental abnormalities associated with these chromosomal aberrations, in that the placental and fetal mass of 21-trisomic infants is similar to that of normal fetuses, whereas those with trisomy 18 are reduced and growth retarded (Creasy *et al.*, 1994).

Although our study does show a significant difference between normal pregnancies and those with an aneuploid fetus, particularly those with trisomy 21, a larger scale study is necessary to determine the true extent of this finding. A clear drawback of the current technology is that it is focused on detecting male DNA sequences, due to the ease of this assay. This deficit could, however, be redressed by examining paternally inherited highly polymorphic sequences, as recently described (Tang *et al.*, 1999). By the application of such developments, it will be interesting to determine whether quantification of fetal DNA levels can be included in an integrated screening approach to improve upon the current low levels of specificity and sensitivity for detecting affected fetuses.

ACKNOWLEDGEMENTS

This work has been supported by the Swiss National Science Foundation (3200-047112.96), the National Institute of Health (Contract Number N01-HD-4-3202) and the German Research Foundation (Tr 452/1-1).

REFERENCES

- Al-Mufti R, Hambley H, Farzaneh F, Nicolaides KH. 1999. Investigation of maternal blood enriched for fetal cells: role in screening and diagnosis of fetal trisomies. *Am J Med Genet* **85**: 66–75.
- Bianchi DW. 1999. Fetal cells in the maternal circulation: feasibility for prenatal diagnosis. *Br J Haematol* **105**: 574–583.
- Bianchi DW, Williams JM, Sullivan LM, Hanson FW, Klinger KW, Shuber AP. 1997. PCR quantitation of fetal cells in maternal blood in normal and aneuploid pregnancies. *Am J Hum Genet* **61**: 822–829.
- Bianchi DW, Simpson JL, Jackson LG, Evans MI, Elias S, Holzgreve W, Sullivan LM, de la Cruz F. 1999. Fetal cells in maternal blood: NIFTY clinical trial interim analysis. *Prenat Diagn* **19**: 994–995.
- Bischoff FZ, Lewis DE, Nguyen DD, Murrell S, Schober W, Scott J, Simpson JL, Elias S. 1998. Prenatal diagnosis with use of fetal cells isolated from maternal blood: five-color fluorescent *in situ* hybridization analysis on flow-sorted cells for chromosomes X, Y, 13, 18, and 21. *Am J Obstet Gynecol* **179**: 203–209.
- Creasy R, Resnik R. 1994. Intrauterine growth restriction. In *Maternal Fetal Medicine: Principles and Practice*, 3rd edn, Creasy R, Resnik R (eds). WB Saunders: Philadelphia; 558–574.
- De La Cruz F, Shifrin H, Elias S, Simpson JL, Jackson L, Klinger K, Bianchi DW, Kaplan SH, Evans MI, Holzgreve W. 1995. Prenatal diagnosis by use of fetal cells isolated from maternal blood. *Am J Obstet Gynecol* **173**: 1354–1355.
- De La Cruz F, Shifrin H, Elias S, Bianchi DW, Jackson L, Evans MI, Simpson JL, Holzgreve W, Klinger K. 1998. Low false-positive rate of aneuploidy detection using fetal cells isolated from maternal blood. *Fetal Diagn Ther* **13**: 380.
- Faas BH, Beuling EA, Christiaens GC, von dem Borne AE, van der Schoot CE. 1998. Detection of fetal RHD-specific sequences in maternal plasma. *Lancet* **352**: 1196.
- Gänshirt-Ahlert D, Börejonsson-Stoll R, Burschky M, Dohr A, Garritsen HSP, Helmer E, Miny P, Velasco M, Walde C, Patterson D, Teng N, Bhat NM, Bieber MM, Holzgreve W. 1993. Detection of fetal trisomies 21 and 18 from maternal blood using triple gradient and magnetic cell sorting. *Am J Reprod Immun* **30**: 194–201.
- Hahn S, Sant R, Holzgreve W. 1998. Fetal cells in maternal blood: current and future perspectives. *Mol Hum Reprod* **4**: 515–521.
- Hahn S, Kiefer V, Brombacher V, Troeger C, Holzgreve W. 1999. Fetal cells in maternal blood: an update from Basel. *Eur J Obstet Gynecol Reprod Biol* **85**: 101–104.
- Hahn S, Zhong XY, Bürk MR, Troeger C, Holzgreve W. 2000. Multiplex and realtime quantitative PCR on fetal DNA in maternal plasma: a comparison with fetal cells isolated from maternal blood. In *Circulating DNA in Plasma*, Stroun M, Anker P (eds). Ann N Y Acad Sci **906**: 148–152.
- Holzgreve W. 1997. Will ultrasound-screening and ultrasound-guided procedures be replaced by non-invasive techniques for the diagnosis of fetal chromosome anomalies? Editorial. *Ultrasound Obstet Gynecol* **9**: 217–219.
- Holzgreve W, Hahn S. 1999. Novel molecular biological approaches for the diagnosis of preeclampsia. *Clin Chem* **45**: 451–452.
- Lo YMD, Corbetta N, Chamberlain PF, Sargent JL. 1997. Presence of fetal DNA in maternal plasma and serum. *Lancet* **350**: 485–487.
- Lo DYM, Tein MSC, Lau TK, Haines CJ, Leung TN, Poon PMK, Wainscoat JS, Johnson PJ, Chang AMZ, Hjelm NM. 1998a. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet* **62**: 768–775.
- Lo YM, Hjelm NM, Fidler C, Sargent IL, Murphy MF, Chamberlain PF, Poon PM, Redman CW, Wainscoat JS. 1998b. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. *N Engl J Med* **339**: 1734–1738.
- Lo YM, Lau TK, Zhang J, Leung TN, Chang AM, Hjelm NM, Elmes RS, Bianchi DW. 1999a. Increased fetal DNA concentrations in the plasma of pregnant women carrying fetuses with trisomy 21. *Clin Chem* **45**: 1747–1751.
- Lo YM, Leung TN, Tein MS, Sargent IL, Zhang J, Lau TK, Haines CJ, Redman CW. 1999b. Quantitative abnormalities of fetal DNA in maternal serum in preeclampsia. *Clin Chem* **45**: 184–188.
- Oosterwijk JC, Mesker WE, Ouwkerk-van Velzen MC, Knepple CF, Wiesmeijer KC, Beverstock GC, van Ommen GJ, Kanhai HH, Tanke HJ. 1998. Fetal cell detection in maternal blood: a study in 236 samples using erythroblast morphology DAB and HbF staining and FISH analysis. *Cytometry* **32**: 178–185.
- Tang NL, Leung TN, Zhang J, Lau TK, Lo YM. 1999. Detection of fetal-derived paternally inherited X-chromosome polymorphisms in maternal plasma. *Clin Chem* **45**: 2033–2035.
- Wald NJ, Watt HC, Hackshaw AK. 1999. Integrated screening for Down's syndrome on the basis of tests performed during the first and second trimesters. *N Engl J Med* **341**: 461–467.
- Zhong XY, Holzgreve W, Hahn S. 2000. Detection of fetal rhesus D and sex using fetal DNA from maternal plasma by multiplex PCR. *Brit J Obstet Gynaecol* **107**: 766–769.